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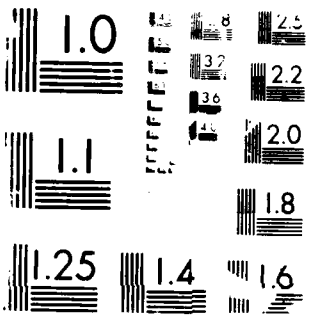
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A New Scientific Instrument for Biogenetic Toxicity Testing (UNCL)
The Fluorescent Cell Viability Counter (FCVC)

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On the basis of analyses of commercial technical information and searches of engineering and scientific data bases concomitant with detailed planning sessions and discussions it has been concluded: (1) a SITS-based system will provide the desired capability of discriminating between living and dead cells enabling the measurement of cytotoxicity, (2) the concept of an automated FCVC is feasible (3) commercial cell sorters cannot perform the functional options ascribed to the FCVC, for the most part, and where this capability is claimed, they cannot perform them as cost effectively and efficiently, (4) the relative simplicity of the proposed FCVC environments and reduce the level of training required for operating personnel. The original design concepts have been modified to include replacement of the original double pass sighting system with a more efficient single pass one, an electrostatically controlled flow chamber, an option for segregation and collection of "resistant" cells for future study and one for the possible detection and enumeration of specific virus particles. In Phase II, it is planned to build a breadboard/demonstration unit of the prototype FCVC. This unit will be used to study the FCVC's principles of operation, to test and choose components and to determine the

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operating parameters of the instrument. This will include the developement of operating standards and quality control protocols.

Phase I Objectives

1. Review various concepts and design options for an electro-optical instrument based on the staining properties of the fluorescent viability indicator, SITS, a cytochemical staining technique.

2. Determine feasibility of SITS-based staining for biogenetic toxicity testing.

3. Evaluate design options for the replacement of existing cell sorter technology with SITS-staining based instrumentation.

4. Compare relative reliability of SITS-based instrumentation with commercially available cell sorters.

5. Identify, evaluate and compare SITS-based technology temporal parameters for cytotoxicity and morbidity measurements with current standards.

6. Identify, evaluate and compare the resolution of SITS-based technology for determination of biogenetic toxicity and morbidity with current technology.

7. Determine feasibility to extending the use of SITS-based instrumentation to field environments remote from laboratories to which cell sorters and human operators must be confined.

Status of the Research Effort

Instrumentation

Engineering concepts and design criteria addressed in the original proposal covered two types of test activities, (1) automatic determination (count) of the ratio of viable to non-viable cells, and (2) preliminary observation and recording of the progressive changes in the fluorescent staining pattern of toxic agent exposed cells over a period of time.

To accomplish this, a flow system and a static type observation system are required. A preliminary system schematic and some very basic operating parameters and design calculations were presented in the initial proposal.

The principal efforts during the first phase of this program were a critical review of the system concept (presented in the light of a literature survey), a clearer definition of the requirements for specific types of system components and the determination of a suitable system operating procedure.

The paradigm used for information gathering and as a basis for comparative judgements, was the cell sorter. Flow cytometers employing closed systems were also examined. A considerable amount of commercial literature, covering various candidate components and subsystems was collected and reviewed. Valuable, detailed insights into the current state-of-the-art were developed by review of texts dealing with cytometry, video microscopy and flow cytometers (Inoué, 1986; Van Dilla, *et al.*, 1985)

From the preliminary design considerations, it appeared evident that, for the system hardware, optimum selections could best be made by accumulating and studying a

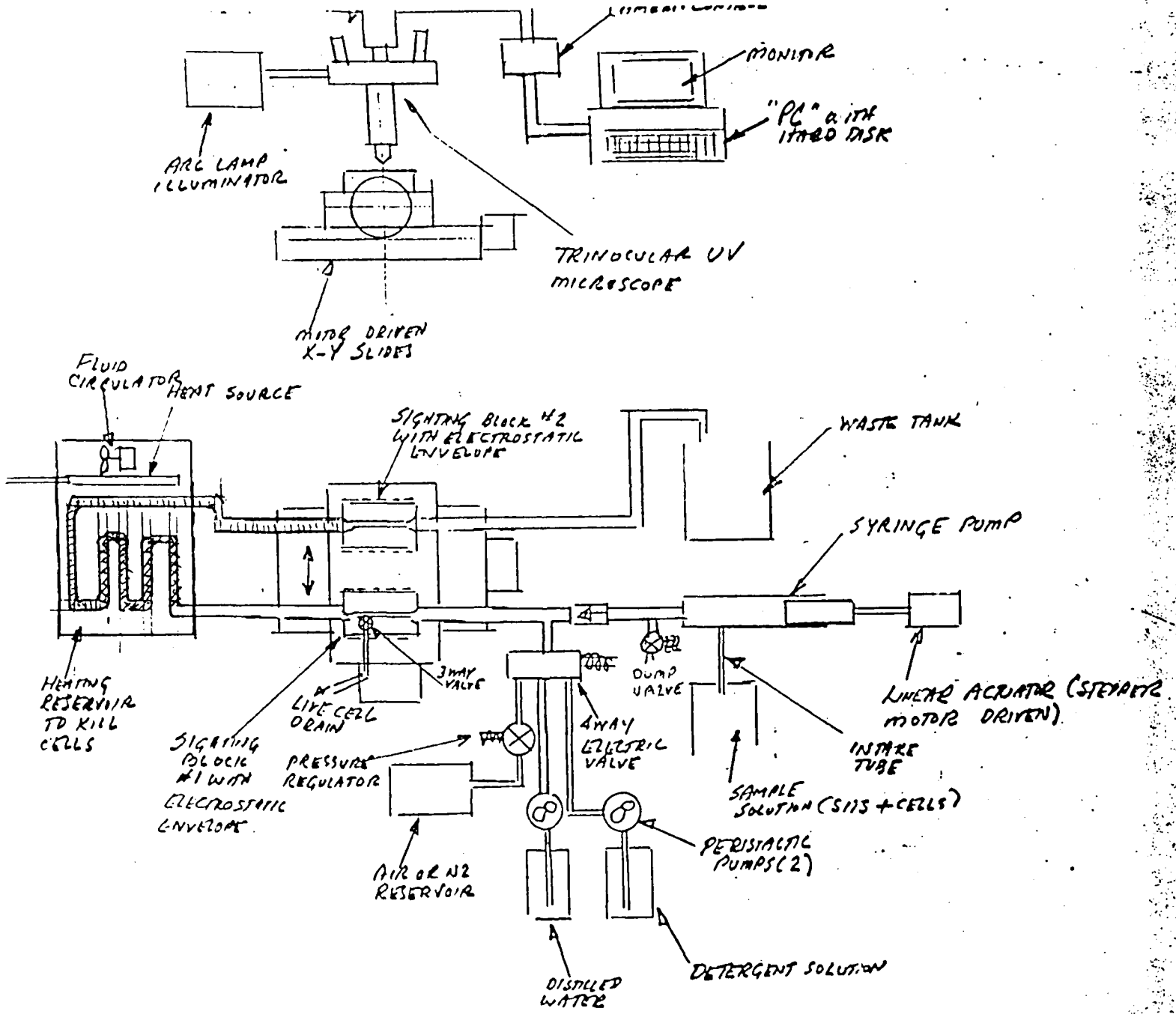

Figure 1. VIABLE/NON-VIABLE CELL DETECTION & COUNTING SYSTEM SC 3/1/88

representative cross-section of data on flow systems, flow control, electronic and other components, microscopes, etc. The literature obtained covered these major areas and a number of sub-divisions of many of the areas. Specifically, as determined by the file labels, catalog and other information fell into the categories listed in Table 1. The "key words" used in our literature search are listed in Table 2.

For our proposed application, there are certain fundamental differences between the instrument we propose and the typical cell sorter. Because our instrument has less stringent technical requirements, it has a correspondingly lower cost. For example, the cell inspection rate is one or more orders of magnitude lower than for a cell sorter.

The initial flow concept in our instrument was a double pass system; i.e., the cell-SITS solution was fed past the sighting station to obtain the initial dead cell count. It was followed by a step during which the cell-SITS solution was fed past the sighting station again, to obtain the total dead cell count. The total viable/non-viable count was then obtained by subtraction. This procedure entailed the use of a number of valves and was determined, upon reflection, to be somewhat cumbersome. Detailed consideration of this problem led to a revision in the design leading to a single pass system. Now, two sighting stations are used and the cell-SITS suspension is pumped directly through and discarded overboard, though (depending on control options) it may have some residence time in a heated, coiled tubing reservoir killing section between the two sighting stations. Included in the design, will be equipment to maintain the cells at their physiological temperature range until they are killed. In order to avoid duplication of the cost of the viewing microscope and attached equipment, it has been decided to slide mount the two sighting blocks and shift the second into place when the count of the total number of (dead) cells is to be made. This slide shifting action will also be used during static testing when sighting on different individual cells or groups of cells. Most likely, a transverse motion slide will also be used to achieve X-Y movement capability.

Further detailed consideration of the system operation led to the selection of a motor driven syringe type feed for the cell-SITS (toxic agent) suspension rather than a peristaltic pump, as in the original proposal. Initially, following a detergent flush, distilled water flush and air purge, the flow system would be dry. The test sample-containing syringe would have sufficient volume to displace the initial slug of the sample through the entire flow system, with the precision displacement under computer control. The accompanying schematic (Figure 1) shows the planned flow system.

The initial displacement of the syringe piston will be sufficient to bring the leading edge of the sample to the check valve just upstream of the sighting block. The piston will then be gradually moved, a controlled distance at a controlled rate, so that a defined volume of sample passes through the sighting block and the dead cells, if any, are counted.

Next, the piston is further displaced so that the sample slug is centered within the heater section of the test tubing. This results in the killing of all the living cells. During this time, the second sighting block is automatically shifted into place, under the microscope. After a predetermined time, the syringe piston is again activated to drive the sample slug through and past the second sighting station so that the total (dead) cell count can be obtained.

With sufficient syringe volume, the test can be repeated. Alternately, the system can be cleaned, flushed, and purged dry, the syringe reloaded with a different sample and a new test performed.

For viewing the cells, a fluorescent, trinocular microscope (with camera tube) will be used. A wide variety of these microscopes exists covering a wide range of capabilities which determine associated costs. A TV-equipped microscope with UV epi-illumination and transmitted tungsten illumination, as manufactured by Olympus and Nikon or Lietz-Wild, appear to be optimum choices.

In our application, the primary function of the microscope is the imaging of the fluorescent cells on the TV camera tube for the dead cell count. By switching to transmitted light, live cells can also be seen. For some applications, it may be desirable to use both light sources.

As described in the original proposal, the intent is to use one line of the raster scan as a long detector and sense the passage of the fluorescent cells across this line. It is expected that the passage of more than one cell at a time will be recognized. Refer to Figure 2.

For this fluorescent application, epi-illumination is generally used with a low wattage (100-200 watt) mercury or xenon arc lamp.

For camera and video processing circuitry (which will count the dead cells), a very wide range of choices exists and an extensive body of literature for available systems covering a broad range of capabilities has been accumulated and reviewed.

The fluorescent emission of the dead cells is in the 400-500 nanometer region of the electromagnetic spectrum with a peak at about 425 nanometers, just at the lower wavelength of blue. Since this wavelength is in a region where the response of most camera tubes falls off, the selection of an optimum tube type depends, to a large extent, on the image intensity at the image plane of the camera. This image intensity can be strengthened by increasing the illumination intensity and by reducing the optical magnification of the image.

The illumination intensity can be increased, to some extent, by using a more powerful arc lamp, though the intrinsic brightness of the arc lamp will establish an upper limit to the illumination level, no matter how powerful the arc. At the same time, however, increases in light scatter in the microscope, and at the object, will tend to reduce image contrast unless more effective blocking filters are used. Blocking filters tend to reduce image intensity.

By decreasing magnification, to provide high image intensity, a concomitant decrease in system resolution is inevitable. This may or may not be tolerable, depending upon the selected scope of image analysis.

From the purely technical standpoint, the short wavelength and probable moderate fluorescence intensity pose no insurmountable problems. But if the use of greater light intensity and lower image magnification proves insufficient, it may be necessary to resort to an image intensified tube. In order to make a rational selection of an optimum tube type (from both the spectral response and economic standpoint) some specific emission intensity data on SITS-dead cell mixes must be obtained.

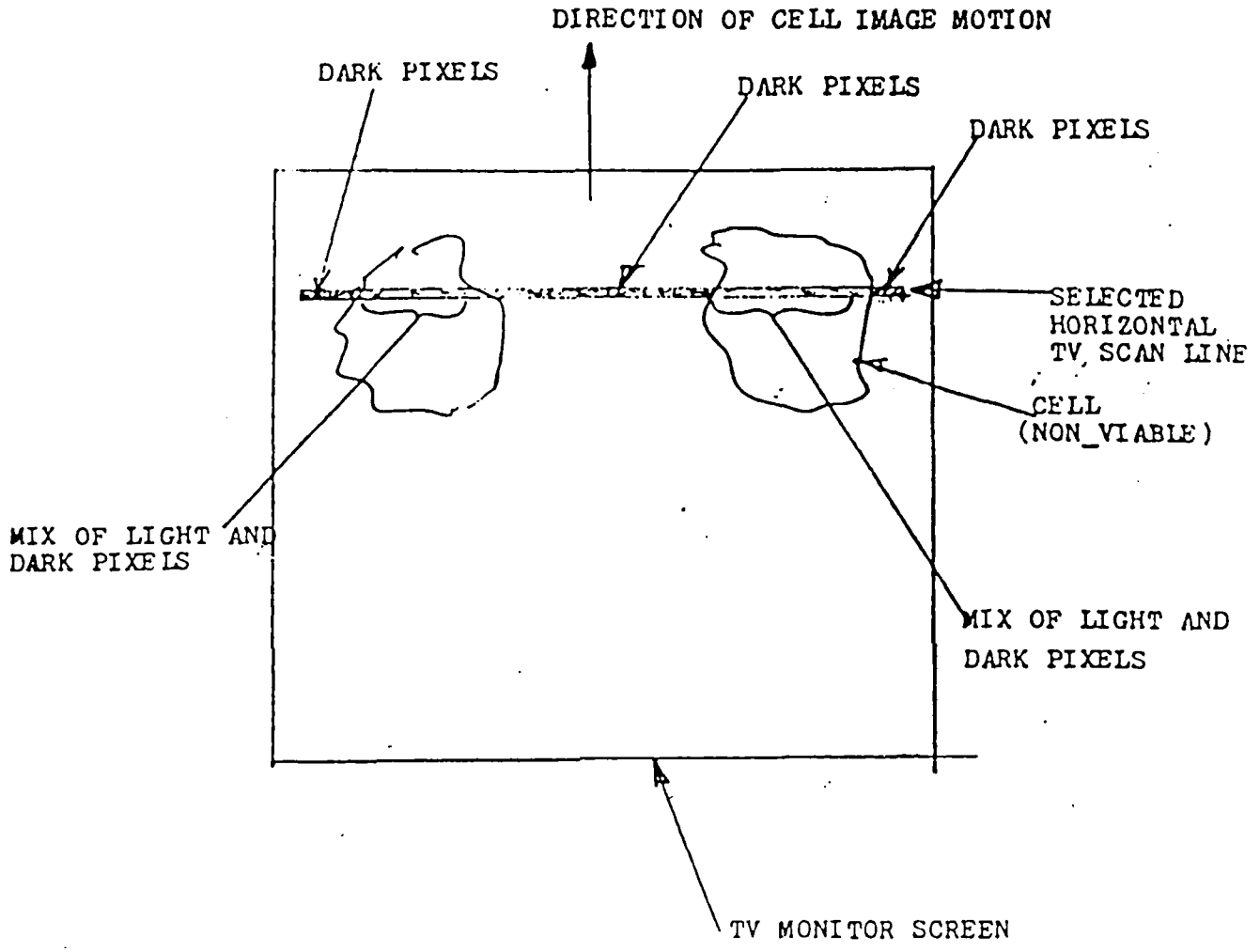

FIGURE 2 NON-VIABLE CELL COUNTING CONCEPT

An initial tube selection could be made by plotting the absolute spectral response and relative costs for several types and selecting an apparent optimum. Alternately, a conservative approach would be to use a camera type currently recommended for fluorescent microscopy.

The text, Video Microscopy, by Inoué lists the sensitivity of selected 1" video cameras and their typical applications in microscopy (Table 7-4 of cited reference). The most sensitive (and most expensive) are the image intensified types used for extremely low light level chemiluminescence, phosphorescence and fluorescence. These are identified as:

I^3 - Vidicon (Zeiss-Venus)	
ISIT (RCA, Sierra, Dage-MTI)	($16,000 + $625 control)
I-Isocon (RCA Cohu)	
I^2 - Newvicon (Cohu)	

Next would be what are qualitatively described as very sensitive types for fluorescence, dark field, high extinction polarization applications. These are identified as:

Isocon (RCA, Cohu)	
I^2 - Vidicon (Zeiss - Venus)	
SIT (Dage, MTI)	($9,370 + $625 control)

Video cameras in a third category described as "sensitive" are not typically used for fluorescent applications (according to the referenced chart) - although with the qualitative indications of relatively high fluorescent output from the SITS stained cells in early experimental work it is possible that this category of camera might suffice. Cameras included are:

MOS (Hitachi)	
Newvicon (Dage-MTI)	($4,450)
Ultricon (Dage-MTI)	($4,450)
Chalnicon (Hammatsu)	($5,800 w/controls)
CID (Panasonic)	

As can be seen from the pricing (in parentheses) presented for several of the cameras, cost rises significantly with sensitivity.

The output of the video camera would be fed to an image processor, a monitor and a VCR or hard disk recorder. Numerous packaged processing systems are available with a wide range of capabilities and costs ranging from $15,000 to $100,000. In this application, the processing/analysis requirements are minimal, and a basic package consisting of a commercial "PC" with added framegrabber board and a video processing board, will suffice. Hardware costs can be limited to a very few thousand dollars (less than $5,000). Programming would be required to perform the dead cell counting function. This would also include digital storing of the image on a disc (an alternate to using a VCR).

A brief description of several"low cost" candidate video grabber-processors follows:

PERX "IN SIGHT" image digitizer DA-2000 series - converts NTSC,EIA-170 or EIA RS-330 video input into digital data. Will save image to disk, print image, difference two images,

compute histogram, etc. Single card installs in IBM PC XT, AT or compatible. Price about $1,500. Additional software available.

PC VISION Frame grabber by Imaging Technology, Inc. - Fluorescence image analysis package available which measures total emitted energy and geometric quantities for fluorescing specimens. For use with IBM PC XT or AT or compatible. Will store to disk. Frame grabber $2,000, software $1,000.

DATA TRANSLATION - DT2853 - 60 HZ Low Cost Frame Grabber - RS170, 330, NTSC inputs $1,595. Real time on-board math and logic operation on single and multiple images. On-board memory for two 512 x 512 x 8 bit images, optional square pixel display, RGB false color output.

Each of the above systems has an RS170 output to drive a monitor.

Additional programming will be required to determine the number of light pixels on the middle raster line so that the dead cell count can be made. This programming will be part of the overall system operating program. Consideration could be given to generating "false color" images to enhance visual observation of the cell image in the static test mode.

From reviewing the literature and discussions of the function of the dead cell counter system under consideration here, it has become evident that the cell sorters which were used as a basis for reference in the original proposal and the FCVC, are really not comparable in function. In the cell sorter, there is emphasis on the high rate of cell passage. Laser illumination is used, with scattering being a primary measurement tool. The sample stream of cells is kept under precise position and velocity control by the injection of sheath fluid around the sample core and the mix is generally dumped overboard (except for systems designed to select and collect specific cell types in the sample).

On the contrary, in our proposed system, cell flow rates are a minimal one or two orders of magnitude less than in a cell sorter, cell positioning (and focus) is not quite as critical.

Therefore, it appears desirable, at this point, to shift from the initially contemplated emphasis, which compared our proposed system with the cell sorter, to a consideration of the FCVC on its own merits.

Potentially, one of the most critical elements in the proposed system is the sighting block/flow-through chamber. The flow path of the cell-SITS solution through the sighting block must satisfy a number of requirements:

a. cells must be in line and separated by a nominal distance,
b. cells must remain in focus,
c. the flow path should be non-clogging over the size range and shapes of all test cell contemplated.

Generally, in commercial cell sorters, a sheath fluid is injected around the sample stream to centralize and channel the cells into an extremely narrow path. Use of sheath fluid in the FCVC would provide control over the cell stream, but at appreciable expense to the system design. It would also introduce a significant problem, since the sheath fluid

would mix ultimately with the sample core, making it impossible to predict the amount of liquid to be checked for dead cells in the second sighting chamber.

As an alternate to the not very desirable (if not impractical) use of sheath fluid to maintain cell stream control, we propose to use electrostatic means. Since cells normally contain some degree of electrical charge, it seems reasonable to expect that by applying a suitable charged field around the sighting block, the cells can be maintained under tight path control. The cell velocity can be controlled by the speed of the carrier fluid which will be in laminar flow. Possibly, an electrostatic control of the forward motion of the cells could also be arranged, if this appears necessary (Sprenger, et al.., 1971).

Because the cells are moving relatively slowly, applying reasonable forces to shift them by an electrostatic field is a practical approach. Consider the following example for lymphocytes: the electrophoretic mobility value is 1.06 μM/sec/V/cm on the average. Assuming, as indicated in the Phase I proposal calculations that a 250 micrometer square flow channel is used, the maximum deflection of a charged lymphocyte is 125 μM. Assume further a nominal electric field of 500 volts per cm. The velocity imparted to the lymphocyte will be 530 μM per second. This means that .24 seconds will be required to bring the lymphocyte from one wall to the center of the flow path. At a lymphocyte velocity of 833 μM per second in the flow direction, the required length of flow path to accomplish centralization is only 200 μM - a very, very small value.

While the above calculation is somewhat simplistic, it serves to point out this inherent practicality of the approach.

When getting down to configuration specifics there is a need for study/analysis and experimentation to evolve a useful detailed design for the electrostatic focusing system.

Ideally, a configuration should be evolved which inherently tends to centralize the flowing cells, in effect an electric analog to the sheath fluid hydraulic action. One such configuration would involve the insertion of an electrode downstream of the sighting point in the microscope objective focal plane to attract the cells. Since the cells will be spaced, it should be possible once to develop a scheme for periodically reversing electrode polarity to detach the cells from the electrode so they flow downstream. While the single electrode provides the simplest, most direct approach, the possibility exists that arrangements of 2 or more electrodes might prove advantageous. For example, with 3 electrodes arranged in an equilateral triangle the possibility exists of the cells being centralized and because of opposing forces not being attracted to any one of the 3 electrodes. This would avoid the problem of the cells sticking to the electrodes.

With respect to some of the other major components/subsystems, the following considerations apply.

Syringe Pump

Factors involved in the selection/design of the syringe pump include:

1. Syringe volume
2. Syringe displacement control
3. Syringe loading
4. Syringe purging/cleaning
5. Syringe replacement

Based upon the preliminary calculations in the Phase I proposal, a representative desired cell concentration of 6×10^4 per ml was obtained. Since only 1000 cells are considered necessary for a statistically significant cell viability determination, the basic syringe displacement volume per sample is 1/60 ml. However, this represents only a small part of the volumetric requirements. At the end of a test cycle, the test sample will be dumped overboard and the test system will be completely filled with the test solution. Therefore, as a minimum the syringe volume will be the sum of the test sample volume (1/60 ml) plus the test system volume. The latter can only be very roughly estimated at this time. It includes:

1. Sighting chamber volume
2. Flow system and thermal chamber tubing volume
3. Flow control component and fitting volumes

As a first gross approximation, the test system volume could be considered equivalent to six feet of 1/16 ID tubing. This equals approximately 3 1/2 ml.

Nominal volumetric displacement rate, again based on Phase I proposal assumptions, is 1/60 of a ml in 300 seconds or .056 microliters/second or 3.4 microliters/minute. The commercially available Harvard Apparatus 50-4910 model pump series provides a unit that appears adaptable to computer control which is well within the range of flow and volume requirements. Both filling and discharge functions are performed.

It is planned to contact Harvard Apparatus in follow-on work to check on the availability of a unit even more specifically suited to the proposed system application. Also additional effort will be made to uncover other suppliers of such equipment. The possibility of a custom design by NSR-EOD using a B&D model 3054 refillable syringe (Cornwall continuous pipetting outfit) with our own design microstepping drive will also be considered.

Cell Killer Unit - This will consist of a liquid filled reservoir with an electric heater rod, thermostat and heater liquid circulating "fan" and a length of tubing for which the sample being checked (with adjacent fluid upstream and downstream) is held during the cell killing period. Approximate exposure is 50°C maximum for up to 40-50 seconds to kill a typical cell. The reservoir will be equipped with a safety valve to guard against over pressure if the thermostat fails to shut off the electric heater current when required. The heated liquid reservoir may have insulation or other protective covering if deemed necessary.

This assembly would be designed and specially fabricated for this application.

Control Computer - The ultimate concept for this system envisions an electronic "black box" with control panel for an essentially automated operation in the viable/non-viable cell determination mode and a partially automated operation in the static test mode. For the breadboard/demonstration system, it appears wiser to use an IBM PC or PC clone with its keyboard to facilitate use of the video capture and processing circuitry and also to facilitate control of the hard disk recorder for the video images. In addition to the keyboard which will primarily perform the function of controlling loading of the system operating programs, making revisions to these programs and controlling the hard disk image capture, a control panel would be provided which will control all the remaining functions which will be of primary interest to the system operator.

Although additional study will be required to fully define the control panel requirements, it can readily be foreseen that some of the controls and indicators will be as follows:

Mode switch to indicate whether the system is to function in the viable/nonviable cell counting mode or the static test mode
Start switch for viable/nonviable cell counting sequence
Indicator lamps to show status of cell counting sequence
Digital readout to show test results
Recycling switch to start new test sequence using same mixture
Indicator lamp to show that insufficient mixture left in syringe pump to run another test sequence
Purge switch to dump contents of syringe pump and flush and blow flow system out
Cell killer temperature indicator and limit warming lights (upper, lower)
Manual control switch to shift sighting block positions
Push button switches to single step and continuous run XY slide steppers
Push button switch to single step and continuous run syringe pump stepper
Limit switch indicator lights for X,Y slide
Limit switch lights for syringe pump piston
Voltage to electrostatic plates of sighting blocks
Power switch and power on light
Automated operation malfunction light(s)
Static inspection mode position store and recall switches and indicators
Other items may be required based on further study.

Although this may appear to be a formidable array of controls and indicators, a number of EOD systems have a comparably complex set. Generally, only a very few controls and indicators are used for normal operation - sometimes only a start button and a cycle complete indicator. By proper grouping and identification of the controls and indicators, confusion can be easily avoided while retaining flexibility of controls essential during diagnostic procedures or specialized operations of the system.

Based on past experience, control panel size for these items will be of the order of 16 x 20 inches.

In order to exercise these control functions it will be necessary to install a (probably) standard I/O card in the computer since each switch and light will require an input or output circuit associated with a particular memory address in the computer.

The control program for the system may be in BASIC or other language depending upon video board requirements and other factors. For some functions such as stepper motor control a lower level language such as Assembler or "C" may be preferable.

Tubing, Valving, Fittings - Flow system components would be made using FDA approved materials such as Tygon® and stainless steel. The valving will be of a type with essentially zero trapped volume and the connecting fittings will be of a similar nature. Valving will be solenoid controlled - either as purchased or as modified by EOD.

During or prior to Phase II, additional data on available flow system components will be obtained to provide a broader range of characteristics currently shown in the available commercial literature.

Microscope - For this application the required choice is a UV microscope with epi-illuminator for fluorescence excitation and tungsten (halogen) transmitted illumination for the viewing the live cells. A trinocular type permitting the attachment of the TV camera is needed. Since the microscope is the primary data gathering element in the system, it was decided to choose a high quality standard unit - such as the Olympus, based on price and familiarity with this instrument.

Further consideration of the microscope selection will be made in Phase II in the light of new data on other makes and evaluation of new viewing requirements or more critical delineation of physical requirements for the unit.

Overall Assembly - Envelope and Installation - It is estimated that the overall flow system with microscope, reservoirs, etc. will fit comfortably on a desk top (2 1/2' x 5') sized table. An auxiliary support table for the computer package and control panel will also be required.

The basic piece of equipment will provide the necessary flow system, visual monitoring (video camera) of the cells, automatic dead cell counter (so that a determination of the initial and final percentages of dead cells in the sample can be made) and, in the static mode, the video system will permit a visual check on the fluorescent pattern changes as the cells die.

A number of options exist which will considerably enhance the functional capabilities of the system. By adding a discharge tube at or just downstream of the first sighting section, along with suitable valving, it should be possible to physically separate live cells from dead cells. Basically, the auxiliary discharge would remain open as the sample slug passes into the sighting chamber. When a dead cell is detected, the auxiliary discharge valve is closed so that the dead cell(s) continues down stream. After a suitable (pre-determined) delay, the auxiliary discharge would be opened again to continue the drain-off and collection of live cells with no further addition of reagents. These cells showing some degree of resistance to toxic substances being studied, could be maintained in culture, for further research.

With the provision of a TV camera and monitor and means to capture the video (most probably a hard disc), interesting possibilities are opened up for further extension of the system capabilities in the sighting mode.

For example, it would be possible to "grab" a picture of a dying cell's fluorescent pattern at spaced intervals (time lapse) and use the hard disc play back for repeated observation and comparison. It would also be possible to determine and record the relative fluorescent intensity at various locations within the cell and also as a function of time during its period of dying.

Although it is expected that the electrostatic control of the sample cell stream would be able to maintain the cells within the focus zone, it is also possible that some amount of manual focusing will be required. This process could also be automated at some expense.

As further extension of the system capabilities, it appears there is a relatively simple method of virus detection and enumeration which lends itself to incorporation in the system. This would involve the use of specific anti-viral antibody-coated microspheres to scavenge virus particles in an effluent. since the identity of the nucleic acid of a specific virus particle is known, the addition of the appropriate fluorescently labelled nuclease

(DNase or RNase) to the mixture, prior to arrival at the sighting station would enable detection/enumeration of detectable quantities of the specific virus adsorbed on the microspheres.

An additional area of interest concerns the use of the FCVC in remote/field environments. Generally, such use implies portability, ruggedness, freedom from excessive supporting auxiliaries such as electric power, air supply, water supply, etc. It may also imply the availability of a stable platform, safety hood, controlled humidity and temperature.

The FCVC can be considered portable, in that its weight and bulk are expected to be quite nominal. Major elements will consist of the "PC" control computer and monitor, the microscope with attached illuminators and video camera, the syringe pumping assembly, and the flow system. Total estimated system weight is below 200 pounds, and system package size should be such that it fits easily on a desk top.

With regard to ruggedness, a number of considerations are involved. The basic flow system consisting of tubing, sighting blocks, some valving and fluid reservoirs, will be inherently rugged and secured to the assembly base plate. The microscope, illuminator and video camera assembly will probably require some additional supporting structures. Some thought has been given to the possibility of a special body for the microscope and camera assembly to provide a more unitized and cleaner package. The computer and monitor will probably require some special mounting, to minimize shock loading, or possibly, ruggedized versions will be used.

Supporting auxiliaries for the system will include: electricity, detergent solution, distilled water and clean air or nitrogen. Electrical power requirements will be quite nominal and can be handled by a portable field generator or by the 12 volt supply from a support vehicle with a 110 VAC inverter. The several fluids mentioned will have to be stored in reservoirs, but the volumes entailed are minor, so that no significant storage or transportation problem is anticipated. When the detailed layout of the system is established, a good estimate of the fluid requirements can be made.

It is not anticipated that any unusual requirements will be imposed on the system support platform stability in the dead cell counting mode. However, if critical visual observations of video digital recordings of the cells in the static observation mode are involved, then a reasonably stable support, comparable to that in the usual laboratory bench situation will be required.

No need for safety hoods is currently foreseen since the flow system is closed. However, safety considerations in sample preparation may lead to the need for safety hoods in some extreme instances. Depending on the nature of the toxic agents being investigated, either an open discharge will be acceptable or a special receiver tank will have to be used. Again, considering the very small liquid volumes anticipated, this should not pose a problem.

With the closed system, humidity is not a factor, in so far as the test samples are concerned. With respect to the optics, however, it is generally recognized that cold optics and high humidity result in condensation on optical elements and a suitable equilibrium temperature must be established and/or proper optical system purging applied when field use is contemplated.

Temperature gradients within optical hardware may also prove to be a problem with regard to localized condensation in the center of lens elements (even interior elements) of vapor evolved from the warmer peripheral areas of the lens. In general, once these problems are encountered and their specific sources identified, they can be solved. With regard to the operational temperature range of the system, there are two major areas of concern: 1) the effect on the computer and 2) the effect on the sample. The general run of "PC's" and "PC" clones are basically normal room temperature devices and field use under environmental temperature extremes may well require ruggedized and consequently much more expensive versions of the computer equipment. If the test system is kept inside a controlled environment vehicle (or tent), this may not be a major consideration.

As for the effect of temperature on the sample, optimal temperature for toxic agent activity must yield to the temperature requirements for maintenance, alive, of the test cells. these must be kept at temperatures which will preserve them, as test systems, in a living and healthy state. Anticipated field test situations must be examined, in detail, from both the hardware and biological standpoints to more definitively determine field system design requirements.

In considering the scope of FCVC instrument design, we analyzed and discussed the possibilities of employing confocal microscopy in image analysis (Dixon and Benham, 1987; Brakenhoff, *et al..*, 1986; Wilson, 1986; White, *et al..*, 1987) and decided that the possible benefits of including this option in the design were far outweighed by the increase in projected cost and complexity.

Possible *in vivo* applications for the FCVC (Damber, et al.., 1986; Feuerstein and Kush, 1986) are under consideration, but must await successful completion of Phase II.

As can be seen by an inspection of the Bibliography, there is an increasing interest in industry, research and clinical medicine in automated analytical microscopy. The FCVC can meet some of the projected needs in these sectors. Furthermore, as often happens, successful marketable instrumentation based on the FCVC can serve as a springboard for the development of a "family" of useful instruments based on the same or similar principles.

Biogenetic Toxicity Measurements with SITS

A major concern in evaluating the suitability of SITS for determination of cytotoxic effects revolves around the question of whether SITS itself is toxic in useful concentrations. All our previous work had indicated that this was not a problem area. However, SITS has become increasingly popular as a "channel blocker" with neuroscientists. This implies a certain amount of toxicity. In our literature search (Table 2) the overwhelming number of references accessed fell into this category of SITS use. The implications of this are that we could have a rather high probability of encountering toxicity in our staining solutions and introducing artefact and error into our experiments.

Since we had not encountered this problem, we were pleased that our results had been supported, to an extent, by the comprehensive work of Horobin, et al.. (1987). As a result of comprehensive chemical analyses of various commercially available preparations and of their own SITS preparation which they themselves had synthesized, they came to the conclusion that much of the work base on SITS as a "channel blocker" had to be discounted. That is to say, that the SITS preparations used were impure, and it was the <u>impurities,</u> not the SITS, which had caused the blocking effect. Furthermore, these workers confirmed our

findings that SITS does not have an affinity for protein. We believe that its affinity for cellular membranes is based on an affinity for lipid. Thus, we felt no need to include the detail of SITS used as a "channel blocker," retrograde label of neurones and/or antibody label.

As can also be seen from Table 2, we reviewed the available literature on fluoresceine diacetate, calcofluor, supravital and vital staining. Although fluorescein diacetate is widely used as a reagent to determine whether cells are viable, its mode of action is such that once the cell has taken it in and the internal chemical reaction has taken place, the cell is both dead and fluorescent. Cells do not die as a result of having taken in SITS. Therefore, fluorescein diacetate is not suitable for the FCVC. Calcofluor, although touted as a stain of viable cells, is highly non-specific and is used as a "specific" stain for fungi. The affinity of this dye for numerous polysaccharide substrates indicates that, in an instrument such as the FCVC, its use could give rise to numerous problems of reliability and specificity.

Publications

A review article on the current status of SITS staining is envisioned. A suitable journal may be Stain Technology or the Journal of Histochemistry and Cytochemistry.

Professional Personnel

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Interactions

New York State matching funds will be used in the development of related instrumentation. We are seeking "bridging funds" from venture capital sources.

Contact has been established with the New Jersey Commission on Science and Technology and a formal notice of intent to submit a proposal for bridge funding has been made. A receptive response was received in telephone conversations and personal contact with commission representatives. The work that would be performed would be in the area of investigation of electrostatic control of the flow path of the cells in the sighting block to assure proper focus. Hopefully these funds will carry our out our research over until we receive Phase II funding. Should we not receive Phase II funding, we plan to accelerate our effort to obtain venture capital. Venture capital support is, of course, expected for the performance of Phase III.

Our bibliographic capability has been enhanced by our having registered in the NASA sponsored LIFENET database.

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TABLE I

Literature - Categories and Contents

1.0 FLOW CELLS
- 1.1 Medical Systems Corp.
- 1.2 Wescor, Inc.
- 1.3 Spectra.

2.0 FITTINGS
- 2.1 Ark-Plas Products, Inc.
- 2.2 Hufmann Engineering Co., Inc.
- 2.3 JACO MFG. CO.
- 2.4 NORTON CO.

3.0 LIQUID LEVEL SENSORS
- 3.1 Thomas Product Ltd.

4.1 TUBING
- 4.1 ZEUS
- 4.2 Ark-Plas Products, Inc.
- 4.3 Norton Co.
- 4.4 Vitro Dynamics, Inc.
- 4.5 National Molded, Inc.

5.0 PUMPS
- 5.1 Cole-Palmer Instrument Co.
- 5.2 Blue White Industries
- 5.3 Arcadia Equipment, Inc. (Tuthill Pump Co.)
- 5.4 Harvard Apparatus
- 5.5 Fluid Metering, Inc.
- 5.6 Manostat
- 5.7 Fluid Metrics, Inc.
- 5.8 Ivek Corp
- 5.9 Randolph Austin Co.
- 5.10 Eldex Laboratories Inc

6.0 CONTROLS (VALVES, ETC.)
- 6.1 Kraft Systems, Inc.
- 6.2 Circle Seal Controls
- 6.3 Hamilton
- 6.4 Micro Pneumatic Logic Inc.
- 6.5 Clippard Instrument Laboratory Inc.
- 6.6 Valcor Engineering Corp.

7.0 FILTERS
- 7.1

8.0 CAMERAS
- 8.1 Illinois Imaging Electronics
- 8.2 Matrix Instruments, Inc.
- 8.3 Panasonic Video Equipment
- 8.4 MP Video, Inc.
- 8.5 Photonic Microscopy, Inc.
- 8.6 Sony Corp. (S.J. Associates, Inc.)
- 8.7 Technical Instruments of Connecticut, Inc.
- 8.8 Symco Inc.
- 8.9 General Electric
- 8.10 Dage MTI, Inc.

9.0 VIDEO MONITORS
- 9.1 Sony
- 9.2 US JVC Corp.

10.0 IMAGE ANALYSIS EQUIPMENT

10.1 Inovision Corporation
10.2 Bioquant R & M Biometrics, Inc.
10.3 Tracor Northern
10.4 Cryo Resources, Ltd.
10.5 Optomax (ITI)
10.6 Bio-Optics
10.7 Brinkmann Instruments, Inc.
10.8 Buehler, Ltd.
10.9 Cambridge Instruments (joint venture with Motion Analysis Corp.)
10.10 Cambridge Instruments
10.11 Drexel Unix Micro Computer Image Analysis System
10.12 Eikonix Corp.
10.13 General Electric Optoelectronic Systems
10.14 Image Technology Corp.
10.15 Le Mont Scientific
10.16 Microsciences, Inc.
10.17 Perceptive Systems, Inc.
10.18 Olympus Corp. (Precision Instruments Div.)
10.19 Photomic Microscopy Inc.
10.20 PERX
10.21 Meridian Instruments Inc.
10.22 Giles Scientific Inc.
10.23 Artek Systems Corp.
10.24 Data Translation Inc.
10.25 Guided Waves Inc.
10.26 Recognition Technology Inc.
10.27 General Imaging
10.28 ADCO Aerospace Inc.
10.29 New Methods Research Inc.
10.30 National Instruments
10.31 Analytical Imaging Concepts

11.0 OPTICAL COMPONENTS

11.1 Rolyn Optics, Co.
11.2 Oriel Corp.
11.3 Ealing Optics
11.4 Boeckler Instruments
11.5 Actron Research Corp.
11.6 Applied Photophysics
11.7 Optical Replication Center, Inc.
11.8 Newport Corporation
11.9 Universe Kogaku l(America), Inc.

12.0 MICROSCOPES

12.1 Bio Rad, Digilab Division
12.2 Reinhardt Instruments (Zeiss)
12.3 Nikon, Inc. Instrument Group
12.4 Tracor Northern
12.5 Seiler Instruments
12.6 Infinity Photo-Optical Co.
12.7 Bausch and Lomb Optical Systems
12.8 A. O. Scientific Instruments
12.9 Ernest Leitz, Inc.
12.10 Meiji Labox America
12.11 Modulation Optics, Inc.
12.12 MS Instrument Co.
12.13 Olympus Corp.
12.14 Spectra Tech
12.15 Schlueter Instruments Corp.
12.16 Titan Tool Supply Co.
12.17 Seiwa Optical Co. Ltd.

13.0 LIGHT SOURCES
- 13.1 Laser Science, Inc.
- 13.2 ABC Electro-Optics
- 13.3 Applied Photophysics
- 13.4 BHK Inc. (Hamamatsu Corp)
- 13.5 Kratos Analytical Instruments
- 13.6 UVP Inc.
- 13.7 Coherent (Laser Products Div.)
- 13.8 Melles Griot

14.0 FIBER OPTICS
- 14.1 Reichert-Jung, Inc. Fiber kOptics Div.
- 14.2 Dolan-Jenner Industries, Inc.
- 14.3 Maxlight Fiber Optics (Div., Raychem Corp.)
- 14.4 Photodyne Inc.

15.0 DIGITAL PANEL METERS AND PRINTERS
- 15.1 Science/Electronics
- 15.2 Acculex

16.0 MISC. RECORDING SYSTEMS

17.0 LASER DISC RECORDERS
- 17.1 Sony
- 17.2 Panasonic

18.0 VIDEO SYSTEMS
- 18.1 Colorado Video
- 18 2 Optomaxine
- 18.3
- 18.4 PMI (Hamamatsu Co.)

19.0 MISCELLANEOUS SENSORS
- 19.1 Service Tectonics Instrument
- 19.2 Gallileo Electro Optics Corp.
- 19.3

20.0 LABORATORY EQUIPMENT
- 20.1 International Light, Inc.
- 20.2 Turner Designs
- 20.3 Service Tectonics Instruments
- 20.4 EM Diagnostic Systems
- 20.5 Ernest F. Fullam, Inc.
- 20.6 Stoelting Research Instruments
- 20.7 Kraft Systems Inc.
- 20.8 LKB Wallac
- 20.9 General Electric
- 20.10 Labindustries Inc.
- 20.11 Narishige
- 20.12 Biorad
- 20.13 Bionique Labs, Inc.
- 20.14 Energy Beam Sciences
- 20.15 Interfacial Dynamics Corp.
- 20.16 Dynalab Corp.
- 20.17 Harrick Scientific Corp.
- 20.18 Advanced Magnetics Inc.
- 20.19 Stoelting
- 20.20 Tri-Continent Scientific Inc.
- 20.21 Medical Disposables

21.0 X, Y POSITIONERS
- 21.1 Narashige, USA, Inc.
- 21.2 Energy Beam Sciences
- 21.3 Burleigh Instruments, Inc.
- 21.4 Oriel Corp.

21.5 Daedal Inc.
21.6 Technical Products International, Inc.

22.0 FLOW CYTOMETERS
22.1 Epics Division of Coulter Electronics
22.2

23.0 MICROBEADS
23.1 Interfacial Dynamics Corp.

24.0 ------

25.0 LIQUID HANDLING & METERING SYSTEMS
25.1 Ivek Corp.
25.2 Dynatech Laboratories, Inc.
25.3 Blue White Industries

TABLE 2

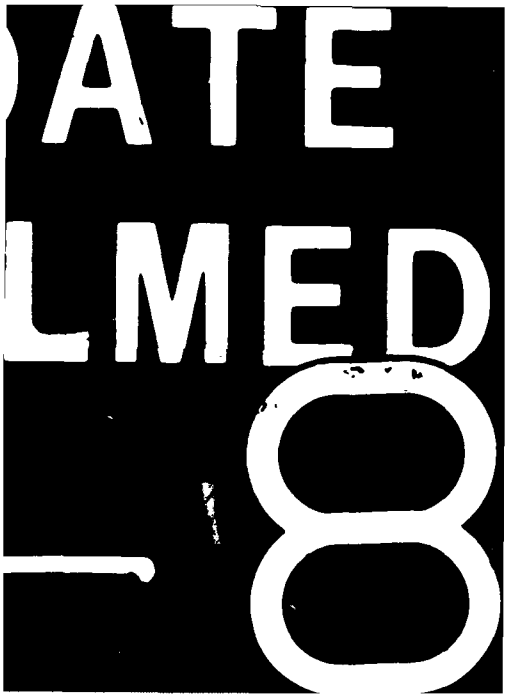
Data bases searched: Medline (1966 - 1988)
Engineering (1975 - 1988)

Key Word	Short Ref.	Long Ref.	Articles Ord.
SITS	594	42	3
Fluoresceine Diacetate	104	26	1
SITS and Flow Cytometry	2	2	2
SITS and Histochemical Implications		1	1
SITS and Cytotoxicity		2	
Microsyringes		3	
Supravital Staining		1	
Flow Cytometry		3	
Vital Staining		3	
Calcofluor	26	18	
Fluoro Gold	1	1	1
Cultured Lymphocytes and Cytotoxicity	12	2	
Liquid Level Sensors	10	3	
Image Analysis		3	1
Flowthrough Chambers	6	6	
Photomultiplier Circuitry	3	3	
Automation in Cervical Cancer Screening and Flow Systems	1		
Fluorsceine Polarimeter and Flow Cytometry	1		
SITS and Cell Sorter	-	-	
Cell Membrane Polarity	60	1	
Video Microscopy	18		

TABLE 2 (continued)

Action Potential	22
Electrical Propagation	20
Electrical Charge	12
Polarity	- -

Note: Major emphasis of this search was on SITS and similar agents, and connected cytochemical techniques.
May 3, 1988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